**Original article**

**Title**

Habitat fragmentation strongly restricts gene flow in endangered ectomycorrhizal fungal populations: evidence from *Rhizopogon togasawarius*, specific to *Pseudotsuga japonica*, across the entire distribution range

**Short title**

Gene flow of an endangered fungus

**Authors**

Hiroshi Abe1\*, Lu Gan1, Masao Murata2, Kazuhide Nara1

1 Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan

2 Akita Forestry Research and Training Center, Akita, Japan.

Hiroshi Abe

Graduate School of Frontier Science, The University of Tokyo

E-mail: [h\_abe@s.nenv.k.u-tokyo.ac.jp](mailto:h_abe@s.nenv.k.u-tokyo.ac.jp)

Lu Gan

Graduate School of Frontier Science, The University of Tokyo

E-mail: [ganlu826@yahoo.co.jp](mailto:ganlu826@yahoo.co.jp)

Masao Murata

Akita Forestry Research and Training Center

E-mail: [muratam0209@gmail.com](mailto:muratam0209@gmail.com)

Kazuhide Nara

Graduate School of Frontier Science, The University of Tokyo

E-mail: [nara@edu.k.u-tokyo.ac.jp](mailto:nara@edu.k.u-tokyo.ac.jp)

**Correspondence**

Hiroshi Abe, Evaluation of Natural Environment Laboratory, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwanoha, 277–8563, Chiba, Japan.

E–mail: [h\_abe@s.nenv.k.u-tokyo.ac.jp](mailto:h_abe@s.nenv.k.u-tokyo.ac.jp)

**Data availability statement**

The *Rhizopogon togasawarius* and *Pseudotsuga japonica* SSR genotypes are available at Dryad Data set (Abe et al., 2024).

https://datadryad.org/stash/share/19UnYaq1-H1j16gkDCaEr\_sbBjaVLEo-JLwhP-IJjqA

**Funding information**

JSPS KAKENHI, Grant/Award Number: 15H02449, 18H03955, and 23H00340

**Conflict of interest disclosure**

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country in which they were performed.

**Abstract**

Habitat fragmentation reduces gene flow, causing genetic differentiation and diversity loss in endangered species through genetic drift and inbreeding. However, the impact of habitat fragmentation on ectomycorrhizal (ECM) fungi remains unexplored, despite their critical roles in forest ecosystems. Here, we investigated the population genetic structure and the demographic history of *Rhizopogon togasawarius*, the ECM fungus specifically colonizing the host tree *Pseudotsuga japonica*, across its entire distribution range (>200 km). These two species are designated as endangered species on the IUCN Red List since they are found only in small, fragmented forests in Japan. We analyzed 236 *R. togasawarius* individuals from five remaining populations across the Kii Peninsula and the Shikoku Island, separated by a sea channel. Simple sequence repeat (SSR) analyses using 20 loci revealed strong genetic differentiation among populations (*F*ST = 0.255), even significant in the nearest population pair separated by a distance of only 8 km (*F*ST = 0.075), indicating extremely limited gene flow between populations. DIYABC-RF analyses implied that population divergence occurred approximately 6000 generations ago between the two regions, and nearly 1500 generations ago between the nearest populations within Shikoku Island, related to past climate events. Because of prolonged genetic isolation, significant inbreeding was confirmed in four of five populations, where effective population sizes became very small (*N*e = 9.0–58.0). Although evaluation of extinction risks for microorganisms is challenging, our conservation genetic results indicated that habitat fragmentation increases extinction risk through population genetic mechanisms, and therefore should not be overlooked in biodiversity conservation efforts.

**Keywords**

Conservation genetics, Demographic history, Endangered microbes, Hypogeous fungi, Microsatellites

# Introduction

Habitat fragmentation is one of the greatest threats to biodiversity (Fahrig, 2003). Restricted gene flow between fragmented habitats causes genetic differentiation of the populations (Frankham et al., 2010) while decreasing genetic diversity in small, isolated populations due to the small gene pool, genetic drift, and inbreeding (Crnokrak & Barrett, 2002; Charlesworth & Willis, 2009; Hedrick & Garcia-Dorado, 2016). Moreover, inbreeding usually increases the homozygosity of deleterious recessive alleles leading to detrimental effects on survival and reproduction, a phenomenon known as inbreeding depression (Charlesworth & Willis, 2009; Frankham et al., 2010; Hedrick & Garcia-Dorado, 2016). These genetic consequences in fragmented populations reduce the fitness of individuals and increase the risks of extinction for populations or species (Young et al., 1996; Lowe et al., 2005; Jump & Peñuelas, 2006). Thus, many studies have investigated the impact of habitat fragmentation on endangered plant and animal species (Frankham, 2015; Willoughby et al., 2015; Schlaepfer et al., 2018; Aguilar et al., 2019); however, no previous studies have focused on its genetic consequences for endangered microorganisms.

Conservation of microbial species is challenging because they cannot be directly observed; therefore, it is difficult to obtain accurate distribution data for evaluations of conservation status (May et al., 2018). Indeed, only 226 fungal species are included in the threatened categories on the IUCN Red List, whereas 24,919 plant and 16,900 animal species are listed as threatened (IUCN, 2023). However, considering that most fungal species play critical roles in terrestrial ecosystems, the conservation of fungal species cannot be overlooked. Among fungal species, ectomycorrhizal (ECM) fungi are crucial components of forest ecosystems that colonize the roots of dominant tree species in temperate to boreal regions (Smith & Read, 2008). Because host trees heavily rely on ECM fungi for nutrient uptake, they cannot survive in environments where compatible ECM fungal partners are absent (Nara, 2006a,b). Although numerous ECM fungi colonize a single tree species, the establishment of seedlings after disturbance can be determined by a few key ECM fungal species (Peay et al., 2009; Glassman et al., 2016; Ishikawa & Nara, 2023). The extinction of such a key fungus would adversely affect host tree establishment and forest regeneration. However, there has been no research regarding the conservation genetics of endangered ECM fungi.

Simple sequence repeats (SSRs, also known as microsatellites) and single-nucleotide polymorphisms (SNPs) are common genetic markers used in population genetic studies of animals and plants. SSR markers are generally multiallelic and neutral, mutating much faster (10 to 100 thousand times) than biallelic SNP markers due to replication slippage and unequal crossover (Ellegren, 2000). Thus, SSRs are more informative than SNPs for species with limited genomic information, where a large number of SNP markers are not available (Guichoux et al., 2011). For example, a direct comparison between SSRs and SNPs in population genetic analyses of a common wood-decaying fungus, *Armillaria cepistipes*, in Europe showed that SSRs were better for detecting finer genetic structures (Tsykun et al., 2017). Labbé et al. (2017) also used 14 SSRs and 27 SNPs as markers for genotyping *Armillaria ostoyae*, but they used only SSR data for demographic history analyses because SNP data exhibited insufficient resolution. Despite these advantages, SSR makers must often be isolated *de novo* for each species. However, the species-specific nature of SSR markers could also be a strength when analyzing environmental samples that contain DNA of nonfocal species.

SSR analyses in ECM fungi, most of which are sexually reproducing diploid organisms as higher plants and animals, were initially used to identify genets (genetically identical units), genet sizes, and genet distribution within a site; some studies used them to analyze the genetic structures of local populations (reviewed by Douhan et al., 2011). Because most spores of ECM fungi are dispersed near the sporocarps, genetic similarity is very high in short-distance genet pairs (e.g., within < 10 m) and decreases according to distance within a local population (Kretzer et al., 2005; Dunham et al., 2006). This restricted dispersal could extend to a larger scale, forming a spatial genetic structure called isolation by distance (IBD), where genetic similarity decreases as geographic distance increases (Wright, 1943). However, population genetics theory suggests that rare long-distance dispersal of spores would enable effective gene flow between distant populations, enabling them to avoid genetic differentiation (Wright, 1969; Bohrer et al., 2005; Bialozyt et al., 2006; Frankham et al., 2010). Although empirical data regarding gene flow in ECM fungi over large geographical scales are scarce, a pioneering study by Vincenot et al. (2012) examined population genetic structures of *Laccaria amethystina*, a common species with a wide host range, across Europe. The authors found minimal genetic differentiation among populations of *L*. *amethystina*, indicating extensive gene flow by wind-dispersed spores on a continental scale over a geological timescale after the last ice age. Similarly, effective gene flow in *Pisolithus microcarpus* over hundreds of kilometers was suggested in eastern Australia (Hitchcock et al., 2011). Sheedy et al. (2015) documented a panmictic population of *Laccaria* sp. over a distance of 300 km in Tasmania. However, they found significant genetic differentiation of this fungus between Tasmania and mainland Australia, or even between eastern and western parts of Victoria. Therefore, gene flow in *Laccaria* sp. may be restricted when its specific host, *Nothofagus cunninghamii*, is not available over large geographical distances.

Compared with these epigeous ECM fungi exhibiting wind-mediated spore dispersal, gene flow could be more restricted in hypogeous ECM fungal taxa that produce belowground sporocarps and depend on mycophagous land animals for spore dispersal. Indeed, Grubisha et al. (2007) found significant genetic differentiation of two *Rhizopogon* species, hypogeous taxa specifically colonizing pine trees, among fragmented populations on two neighboring small islands in California, indicating limited spore dispersal between the populations, even on the scale of several kilometers. Abe et al. (2018) also identified four genetically different *Rhizopogon roseolus* populations corresponding to geographical regions in Japan. However, no previous studies examined gene flow and genetic differentiation in hypogeous ECM fungi across the entire distribution range, partly because of difficulty in locating and collecting sufficient hypogeous sporocarps for population analyses.

*Rhizopogon togasawarius* (Mujic et al., 2014) is a specific ECM fungal symbiont to *Pseudotsuga japonica*, an endangered conifer tree (EN on the IUCN Red List, IUCN, 2023) endemic to Japan. A molecular phylogenetic study indicated that this symbiotic partnership coevolved for over 30 million years after the comigration of North American ancestors (Mujic et al., 2019). Due to the extensive transformation of natural forests to the plantations of *Cryptomeria japonica* and *Chamaecyparis obtusa* in the late twentieth century in Japan (Mori & Kumazaki, 1990), the distribution of *P. japonica* has significantly decreased from previous natural distribution records, now limited to specific preservation areas with approximately 2000 remaining trees (Tamaki et al., 2018; IUCN, 2023). Because *R. togasawarius* is only compatible with *P. japonica* under natural conditions (Murata et al., 2017), its distribution range is restricted to the distribution of the host tree (Okada et al., 2022). Therefore, similar to the endangered host *P. japonica*, *R. togasawarius* has been newly classified as an endangered species (EN on the IUCN Red List, IUCN 2023). Tamaki et al. (2018) found significant genetic differentiation of the host *P. japonica* populations, indicating a threat of inbreeding depression in small, isolated forests. Greater threats could be expected for *R. togasawarius* because *Rhizopogon* spores are dispersed by mycophagous land animals (Vašutová et al., 2019; Borgmann-Winter et al., 2023), the migration of which is confined to short distances and severely affected by geographical barriers (Kretzer et al., 2005; Grubisha et al., 2007; Abe et al., 2018). Using the extremely fragmented habitats and the well-defined distribution range of *R. togasawarius*, it may be possible to investigate population genetic structures and gene flow in this microorganism across its entire distribution range, providing the first conservation genetic insights about an endangered microbial species.

Here, we investigated the population genetic structure of the ECM fungus *R. togasawarius* across its entire distribution range, constituting the first conservation genetics study of an endangered microbial species. This study examined three hypotheses: 1) whether the forest fragmentation of *P. japonica* restricts gene flow in its specific ECM symbiont, *R. togasawarius*, across populations; 2) whether the potentially restricted gene flow resulted in lower genetic diversity and higher inbreeding within each *R. togasawarius* population; and 3) whether the genetic differentiation of *R. togasawarius* populations corresponded with differentiation of the host populations. The results presented here provide key insights into the conservation of microbial species, as well as the conservation of forest ecosystems, considering their intimate relationships with endangered host trees.

# Materials and methods

## Study site

This study was conducted in five *P. japonica* forests located in warm temperate areas of Japan: Kawamatakannon (KW), Sannokogawa (SN), and Ohmata (OM) on the Kii Peninsula, and Yasudagoyama (YS) and Nishinoko (NK) on Shikoku Island, with distances between them ranging from 7.8 km to 207.5 km (Table 1, Figure 1b). Our study sites represent all major populations and cover the entire geographical range of *P. japonica*. The Kii Peninsula forests (KW, SN, and OM) are separated from the Shikoku Island forests (YS and NK) by the Kii Channel. These forest patches are surrounded by Japanese cedar and cypress plantations established in the 1950s, where *P. japonica* coexists with *Tsuga*, *Abies*, and *Quercus* species. Additional site details were provided by Okada et al. (2022).

## Population sampling and DNA extraction

Sporocarps are usually used for sampling in population genetic studies of ECM fungi (Douhan et al., 2011). However, this approach is feasible only for common ECM fungal species, as fungal fruiting is seasonally limited and influenced by environmental factors (Douhan et al., 2011). Because *R. togasawarius* has only two records of sporocarps (Mujic et al., 2014; Orihara, 2019), corresponding to undetectably infrequent ECM colonization on resident trees (Murata et al., 2013), population sampling via sporocarps would be unfeasible. Instead, *R. togasawarius* is dominant in soil spore banks and plays significant roles in host seedling establishment after disturbance (Murata et al., 2017; Okada & Matsuda, 2022), similar to other *Rhizopogon* species (Buscardo et al., 2010; Kipfer et al., 2011; Glassman et al., 2016). Therefore, approaches that combine systematic soil core sampling and bioassays to bait spore banks would overcome the difficulty of population sampling for *R. togasawariu*s. Grubisha et al. (2007) partially used this method and confirmed no significant difference in allele frequencies compared to those of sporocarp sampling.

We collected 20 soil cores (5 × 5 cm to 10 cm in depth after removal of organic soil layers) near randomly selected *P. japonica* trees in each of KW, SN, and OM forests in June 2015. For YS and NK forests on Shikoku Island, we utilized randomly selected soil core samples that had been collected in August 2015 in a previous study using the same method (Okada et al., 2022). These soil samples were subjected to bioassays (see below) to bait *R. togasawarius* individuals. The geographical positions of the sampled soil cores were recorded using GPSMAP 64SJ (Garmin).

Before bioassays, soil samples were subjected to removal of organic debris and small stones, then air-dried in paper bags for 2 months at room temperature. Each dried soil sample was placed in three replicate 50-mL centrifuge tubes with drainage holes containing 25-mm cotton balls at the bottom. Soil-filled tubes were subjected to a 1-h heat treatment at 70°C in a dry-heat oven (MOV-212P2; Sanyo) to deactivate other fungal mycelium and spore propagules and effectively bait the heat-tolerant *R. togasawarius*, in accordance with the procedure of Murata et al. (2017)*.* Additionally, three negative controls filled with sterilized mixtures of several field soil samples (120°C for 20 min) were prepared for each sampling site. Considering the lack of available seeds for *P. japonica*, *Pseudotsuga menziesii* served as the substitute bioassay host plant, as in the work of Murata et al. (2017). A single germinating seed (pretreated with 5% NaClO solution for 10 min for surface sterilization) was planted in each bioassay tube. The tubes were then incubated for 3–5 months in growth chambers (MLR-351; Sanyo) with a light/dark cycle comprising 16 h of light at 25°C and 8 h of darkness at 20°C; watering was conducted every 2–5 days, as needed.

After the bioassay periods, one to five ECM root tips of *R. togasawarius* on each seedling, depending on availability, were collected under a dissecting microscope (SZ2; Olympus) based on distinct characteristics, such as thick white mantle with black-pigmented spots (typical of the subgenus *Villosuli*, Molina et al., 1999) and abundant rhizomorphs (Okada et al., 2022). These collected root tips were stored separately in 2-mL tubes with 100 mL of CTAB buffer for subsequent DNA extraction. Total DNA was extracted by a modified version of the CTAB method (Nara et al., 2003), dissolved in 50 µL of TE buffer, and stored at −30°C for further analysis.

We also collected host tree samples at the same locations on the dates of soil core sampling to analyze the genetic structures of the host populations. Fresh needles or root cambium tissues were obtained from 19, 19, 19, 20, and 17 trees in KW, SN, OM, YS, and NK, respectively (Table 1); preserved with silica gel at 4°C; and used for DNA extraction as described above for ECM root tips.

## SSR genotyping

To analyze the population genetic structures of *R. togasawarius*, we used 20 polymorphic SSR markers (Table 2), including two identified from other *Rhizopogon* species (Kretzer et al., 2000, 2004) and 18 newly developed markers. Of the new markers, six were designed by dual-suppression-PCR (Lian & Hogetsu, 2002) and 12 were designed from our draft genome data generated using an Illumina iSeq 100 system (Illumina). Further information regarding marker development and selection is presented in Method S1.

SSR loci were coamplified by multiplex PCR using universal tail primers for fluorescent labeling, in accordance with the method described by Blacket et al. (2012). PCR was conducted in 10 µL of reaction solution consisting of 5.0 µL of either AmpliTaq Gold 360 Mix (Thermo Fisher Scientific) or Emerald Amp PCR Master Mix (Takara Bio), along with 0.06 µL of 20 mM forward primer with tailed sequences, 0.08 µL of 20 mM tail primer with different fluorescent labels (FAM, HEX, NED, and PET), 0.08 µL of 20 mM reverse primer, and 1.0 µL of DNA template in 200-μL tubes. The PCR cycling conditions were established as previously described (Abe et al., 2017). For fragment analysis, 1.0 µL of a 100-fold diluted PCR product was mixed with 0.875 µL of Hi-Di formamide (Thermo Fisher Scientific) and 0.025 µL of GeneScan 600 Liz Size Standard (Applied Biosystems). After a 3-min heat shock at 94°C, capillary electrophoresis was performed using a 3730xl DNA Analyzer (Applied Biosystems). Fluorescence peaks corresponding to allele sizes were analyzed using Peak Scanner v.1.0 (Applied Biosystems). To avoid the repeated inclusion of genetically identical individuals in subsequent analyses, we excluded redundant samples with identical genotypes at all loci. Additionally, we excluded samples that differed at only one of the 20 loci to avoid misidentification based on somatic mutations or scoring errors (Arnaud-Haond & Belkhir, 2007). All of these redundant samples originated from the same bioassay tubes, indicating that the same vegetative mycelium had colonized multiple ECM root tips in the tube.

We also genotyped host individuals using the same method with six polymorphic SSR markers (*PmOSU\_2D4*, *PmOSU\_3H4*, *PmOSU\_4E9*, *BCPsmAG12*, *BCPsmAG23*, and *BCPsmAG37*), which were originally developed for *P. menziesii* (Amarasinghe & Carlson,, 2002; Slavov et al., 2004) and were also implemented for genotyping of *P. japonica* (Tamaki et al., 2018) (Table S1).

## Population genetic analysis

The population genetics statistics of *R. togasawarius*, including the effective number of alleles (*A*e), observed heterozygosity (*H*o), and expected heterozygosity (*H*e), were calculated with GenAlex 6.5 (Peakall & Smouse, 2012). Allelic richness (*A*r) and inbreeding coefficient (*F*IS) were determined using FSTAT 2.9.3.2 (Goudet, 1995), with deviation of *F*IS from zero tested through 2000 randomizations in each population.

The population structure of *R. togasawarius* was analyzed by Bayesian clustering in STRUCTURE 2.3.4 (Pritchard et al., 2000). Ten independent runs of 100,000 Markov chain Monte Carlo iterations each (*K* = 1–9) with 10,000 burn-in iterations were conducted. The number of clusters (*K*) was determined using the Delta *K* method (Evanno et al., 2005) and the posterior probability (LnP (*K*)) (Pritchard et al., 2000) in the R package POPHELPER v2.3.1 (Francis, 2017), which was also used to align runs and visualize the outcomes. Individual-based principal coordinates analysis (PCoA) was performed in GenAlex 6.5 using the genetic distance among SSR genotypes. Multivariate analysis of variance (MANOVA) in R version 4.2.2 (R Core Team, 2022) was used to examine the PCoA scores (first two principal component axes) across all five populations. A neighbor-joining (NJ) tree among the five populations of *R. togasawarius* was constructed using POPTREE2 (Takezaki et al., 2009), based on Nei’s *D*A distance (Nei et al., 1983) with 1000 bootstrap replicates. Phylogenetic network analysis of *R. togasawarius* individuals was conducted in SplitsTree version 4.19.1 (Huson, 1998; Huson & Bryant, 2005) using the neighbornet method. To assess genetic differentiation among populations, pairwise and total *F*ST, as well as the standardized *G*′ST (Hedrick, 2005), were calculated by GenAlex 6.5 with significance tests that used 999 permutations. The correlations between these genetic differentiation indices and geographical distance were analyzed using the Mantel test in GenAlex 6.5.

The above population genetic statistics were also calculated for the host *P. japonica* and compared with those of *R. togasawarius*. Using the pairwise *F*ST or *G*′ST matrices among the five populations, we analyzed the correlations of population genetic differentiation between the host and ECM fungus using the Mantel test. Because the number of SSR loci differed between the host and ECM fungus, comparisons of these population statistics and the correlation test were also performed after the SSRs of *R. togasawarius* had been rarefy to six (i.e., identical to the host) with 10,000 resamplings using R. STRUCTURE analyses were also applied to the host populations as in the analyses of *R. togasawarius*, except the LOCPRIOR admixture model was used because the default settings could not detect significant population structure at any K value for the host (Figure S1c), probably due to low divergence (Hubisz et al., 2009).

## Demographic history

The effective population size (*N*e) of *R. togasawarius* was calculated using NeEstimator V2.01 (Do et al., 2014), with the smallest sample size (*n* = 18) and a minimum allele frequency of 0.05 as cutoffs, in accordance with the recommendations of Waples and Do (2010). These values served as appropriate thresholds to exclude singletons in our data set. Past genetic bottleneck events within populations were estimated using BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996) with a two-phased model (T.P.M), as well as a 95% single-step mutation rate and a variance of 12 for multiple steps, in accordance with the recommendations of Piry et al. (1999); the heterozygosity excess was examined using Wilcoxon’s signed-rank test.

We performed Approximate Bayesian Computation (ABC) with supervised machine learning using DIYABC Random Forest (DIYABC-RF) version 1.0 (Collin et al., 2021) to explore the demographic history of *R. togasawarius*. We excluded five individuals based on the results of STRUCTURE analysis (*K* = 5, q < 0.90) due to low assignment accuracy or potential admixture because the ABC framework requires populations without continuous gene flow (a total of 231 individuals).

We used the model choice feature in DIYABC-RF to determine the best-supported scenario because multiple scenarios were inferred from the results of the NJ phylogeny (Figure 2a). We considered two potential coalescence scenarios (Scenario 1–2, Figure 2b) then selected the optimal divergence topology. We did not include bottleneck scenarios in our ABC analysis because no bottleneck wes detected (Table 4). During model selection, we generated 3000 training data sets for each scenario with a uniform prior distribution of parameters and divergence times (*t*4 > *t*3 > *t*2 > *t*1). Prior parameters and sex ratio were set to default. The scenario choice and posterior probability estimation of the best-supported scenario were performed using a random forest that consisted of 1000 trees with five independent runs. The scenario choice and prediction quality were evaluated using prior error rates. We also visually checked how well the observed datasets fit to the simulated datasets when projected onto the linear discriminant analysis (LDA) axis (Collin et al., 2021).

For parameter estimation, we generated a training data set that consisted of 20,000 simulated data sets from the best-supported scenarios (Scenarios 1) using partial least squares (PLS) regression analysis to explain 90% data variance. Point estimates and global/local accuracy indices (normalized mean absolute error, NMAE) were computed using out-of-bag estimators with 10,000 data points, and median values were used to summarize the point estimates (Collin et al., 2021). The parameter estimation process involved a random forest with 1000 trees and five independent runs.

We used DIYABCskylineplot 1.0.1 (Navascués et al., 2017) to track the demographic history of each *R. togasawarius* population. The parameters for the analysis were set as follows: num\_of\_points = 100 (number of points to draw the skyline plot), max\_num\_of\_periods = 50 (maximum number of periods to simulate), prior\_THETA\_min = 0.001 and prior\_THETA\_max = 1000 (THETA: population size measured by 4*N*eμ, where *N*e is the effective population size and μ is the mutation rate per generation), prior\_GSM\_min = 0.1 and prior\_GSM\_max = 0.8 (GSM: generalized stepwise mutation model for SSRs), prior\_TAU\_max = 5 (maximum time, measured in number of mutations per locus), and prior\_MUTRATE\_min = 0.0001 and prior\_MUTRATE\_max = 0.001 (MUTRATE: μ value). All other options and priors were set to default.

# Results

## Multilocus genotypes of *R. togasawarius*

We collected a total of 391 root tips colonized by *R. togasawarius*: 69, 62, 21, 62, and 177 from KW, SN, OM, YS, and NK, respectively. SSR analyses identified 236 multilocus genotypes (MLGs): 53, 44, 18, 35, and 86 genotypes from KW, SN, OM, YS, and NK, respectively. Multiple MLGs were frequently observed within a single bioassay tube, with an average of 2.1 MLGs per tube.

## Genetic diversity and differentiation of *R. togasawarius* populations

The genetic diversity indices (*A*e, *H*o, *H*e, and *A*r) for *R. togasawarius* were consistently lower in the Kii region (KW, SN, OM) than in Shikoku Island (YS, NK), except for *A*r in SN (Table 3). The effective number of alleles (*A*e) ranged from 1.74 (KW) to 2.52 (NK) across study sites (Table 3). Observed heterozygosity (*H*o) and expected heterozygosity (*H*e) ranged from 0.324 (KW) to 0.476 (YS) and 0.364 (KW) to 0.524 (YS), respectively (Table 3). The highest allelic richness (*A*r) value (4.00) was observed in NK, whereas the lowest *A*r value (2.94) was observed in KW (Table 3).

High inbreeding coefficients (*F*IS) were observed, with values ranging from 0.049 (NK) to 0.120 (KW); four of the five coefficients were statistically significant after Bonferroni correction (*P* < 0.05) across study sites (Table 3). Notably, all genetic diversity statistics were lowest in the KW, corresponding with the highest *F*IS.

In STRUCTURE analyses, the highest and second-highest delta *K* values were observed at *K* = 2 and 5, respectively. LnP(*K*) values reached a plateau at *K* = 5 (Figure S1a). These results indicated the presence of two distinct clusters representing the Kii Peninsula and Shikoku Island, followed by five subclusters corresponding to the studied sites (Figure 1a). STRUCTURE bar plots at other *K* values were also provided as Figure S2a). PCoA and subsequent MANOVA (Figure 1c, PC1: 19.15%, PC2: 10.35%) also supported this population differentiation (*P* < 0.01). The total *F*ST and *G*′ST values among all sites were 0.255 and 0.502, respectively, indicating strong genetic differentiation among *R. togasawarius* populations. Significant values of *F*ST (ranging from 0.075 to 0.200) and *G*′ST (ranging from 0.208 to 0.540) were found in all pairs of *R. togasawarius* populations, even within the Kii Peninsula and Shikoku Island regions (Table 5). *F*ST and *G*′ST values among *R. togasawarius* populations tended to be correlated with geographical distance, but these correlations were not significant according to the Mantel test (Figure 3a, *F*ST: *R*2 = 0.348, *P* = 0.141, *G*′ST: *R*2 = 0.364, *P* = 0.150). The NJ tree among *R. togasawarius* populations also reflected major differentiation between Kii Peninsula and Shikoku Island populations, as well as subdivisions within each region (Figure 2a). The phylogenetic network tree of *R. togasawarius* showed the same topology as the NJ tree (Figure 1d).

## Demographic history of *R. togasawarius* populations

The effective population size was smallest for the KW with *N*e = 9.0 (5.4–15.2; 95% confidence interval [CI]), followed by the other two sites in the Kii Peninsula region (Table 4). Two sites in the Shikoku region had higher *N*e values of 30.3 (YS) and 58.0 (NK), but the values remained small. Bottleneck analyses identified no significant bottleneck events in the past for any of the sites (Table 4).

DIYABC-RF analysis revealed that Scenario 1 was the best-supported scenario, indicating that SN and KW diverged at *t*2 after OM had diverged from their ancestor at *t*3 in the Kii Peninsula. The posterior probability and prior error were 0.722 and 0.185, respectively (Table 6a). The density plot on the LDA axis also showed better support for Scenario 1 (Figure S3). Parameter estimation for *t*1, *t*2, *t*3, and *t*4 yielded median values of 1512.2, 3248.2, 4969.0, and 6038.3, respectively (Table 6b). Their CI and NMAE values are summarized in Table 6b. DIYABCskylineplots for individual *R. togasawarius* populations detected no clear trends of population expansion or contraction in the past, while SN and NK exhibited slight population contractions recently (Figure S4).

## Correlation with host population genetic structures

SSR analysis of *P. japonica* populations revealed a genetic diversity distribution pattern similar to that of *R. togasawarius* across all sites. Observed and expected heterozygosity (*H*o and *H*e, respectively) ranged from 0.500 to 0.683 and 0.559 to 0.628, respectively; they were consistently higher in the Shikoku populations (YS, NK) than in the three Kii populations (KW, SN, OM) (Table S2). The number of effective alleles and allelic richness (*A*e, *A*r) ranged from 2.64 to 3.35 and 4.67 to 5.35, respectively; the values were highest at SN and lowest at KW (Table S2). Upon comparison of the extent of genetic diversity statistics between the symbionts, *R. togasawarius* populations exhibited consistently lower genetic diversity relative to *P. japonica* populations at the same sites, even after fungal SSR loci had been rarefied to the same number (Table S2).

Genetic differentiation (*F*ST and *G*′ST) among the *P. japonica* populations was significant, as confirmed in a previous study (Tamaki et al., 2018), although the differentiation levels were significantly lower in the host population than in the fungal population (Figure S5, Table S3). The correlations of population genetic differentiation (*F*ST and *G*′ST) values between *R. togasawarius* and *P. japonica* were strong, explaining more than 30% of variance in the data set (Figure 3b, *F*ST: *R*2 = 0.390, *G*′ST: *R*2 = 0.324). The correlation of *F*ST values between the host and fungal populations was statistically significant according to the Mantel test, whereas that of *G*′ST was marginally significant (Figure 3b, *F*ST: *P* = 0.032, *G*′ST: *P* = 0.065). These results were consistent even after the SSR rarefaction (Figure S7). The topology of the *P. japonica* NJ tree was identical to that of *R. togasawarius* (Figure 2a, Figure S6), despite the lower branching support of the host tree.

# Discussion

## Restricted gene flow among *R. togasawarius* populations across the entire distribution range

Significant genetic divergence of the fragmented *R. togasawarius* populations clearly indicated that its gene flow was strongly restricted by fragmentation, supporting our Hypothesis 1. Spores of hypogeous *Rhizopogon* species are usually dispersed by mycophagous land animals (Luoma et al., 2003; Vašutová et al., 2019; Elliott, 2022), and geographic barriers affect their migration (Kretzer et al., 2005; Grubisha et al., 2007; Abe et al., 2018). The endangered *P. japonica* forests are separated into two geographic regions by a sea channel, then separated by steep ridges in different mountainous areas within each region. Although mycophagous animals feeding on *R. togasawarius* have not been identified, such animals may be unable to move across these geographic barriers. In previous studies, Grubisha et al. (2007) identified substantial genetic differentiation among *Rhizopogon* populations (*R. vulgaris*; *F*ST = 0.054, *R. occidentalis*; *F*ST = 0.258) in Northern Channel Islands that were separated by a small sea channel (5 km) or even a dry valley within an island. Conversely, epigeous *Suillus* species, a closely related genus with wind-mediated spore dispersal, exhibited minimal genetic differentiation across hundreds of kilometers (*Suillus spraguei*; *F*ST = 0.00–0.15, *S. luteus*; *F*ST = 0.00–0.06) (Rivera et al., 2014; Pildain et al., 2021). Therefore, the impact of habitat fragmentation on spore dispersal, and thus gene flow, would be greater in hypogeous fungal taxa than in epigeous fungal taxa.

The results of demographic history analyses revealed that genetic divergence among *R. togasawarius* populations spans thousands of generations. Although the exact generation time of *R. togasawarius* is unclear, the fossil records of the host tree species could facilitate estimation of the divergence time. During the last glacial period, *P. japonica* thrived in lowlands in eastern Shikoku Island and the Kii Peninsula, as indicated by pollen fossil records (Nakamura & Katto, 1953; Shimakura, 1969; Ooi, 2016). In the post-glacial era, the distribution of *P. japonica* shrunk to steep mountain ridges in the context of competition with other warm temperate conifer and evergreen broadleaved trees. Therefore, the most recent differentiation of *R. togasawarius* (YS and NK for 1512.2 generations ago) may correspond to host isolation near the end of the last glacial maximum (19,000 years ago). In this scenario, the generation time of *R. togasawarius* becomes 12.6 years, which is similar to the estimated generation time for other ECM fungi (around 17 years; Dahlberg and Mueller, 2011). Therefore, the divergence between the two regions could have occurred around 76,000 years ago when they were connected by a landmass during the ice age but separated by a large river (Yashima, 1994), suggesting fragmentation likely resulted from past climate changes (e.g., ice ages) and associated biogeographic factors predating anthropogenic impacts.

## Genetic diversity and inbreeding within fragmented *R. togasawarius* populations

Prolonged isolation in fragmented habitats resulted in the loss of genetic diversity in *R. togasawarius* compared with other common *Rhizopogon* species. For example, heterozygosity values in our study were lower than that of the common *Rhizopogon* species in Japan (*R. roseolus*: *H*o = 0.438–0.612, *H*e = 0.611–0.696; Abe et al., 2018). Similarly, expected heterozygosity (*H*e) values in *Rhizopogon vinicolor* and *Rhizopogon vesiculosus*, widely distributed species of the subgenus *Villosuli* in North America, were 0.50 ± 0.07 and 0.50 ± 0.07, respectively (Dunham et al., 2013). Although Grubisha et al. (2007) reported lower heterozygosity in small island populations of *R. occidentalis* (*H*e = 0.07–0.26, *H*o = 0.06–0.24) and *R. vulgaris* (*H*e = 0.40–0.46, *H*o = 0.31–0.47), this was probably due to the founder effect on the island. Because these two *Rhizopogon* species are nonthreatened species with large distribution areas in western North America, their mainland populations would have much higher genetic diversity. Although all of these studies used randomly selected SSR markers within the same genus, direct comparison of heterozygosity values should be cautious when analyses are based on different loci (Väli et al., 2008; Queirós et al., 2015)

The reduced genetic diversity in *R. togasawarius* populations is clearly indicated by the small effective population size, too. Effective population size is the idealized population size after adjustment for the observed genetic diversity loss within the population; it is one of the most important parameters in conservation genetics for plants and animals. Although comparable *N*e data are scarce for fungi, a much larger size was estimated for a local population of *A. ostoyae* (a basidiomycete pathogen on pine roots) in southwestern France (*N*e = 876 –3150; Labbé et al., 2017). The scarcity of fungal *N*e data is due to the difficulty in sampling fungal populations, as well as the difficulty of developing polymorphic SSR markers for fungi (Dutech et al., 2007), which have smaller genome sizes and therefore fewer SSR loci than plants and animals (Mohanta & Bae, 2015). Here, we utilized 20 SSRs, the highest number in mycorrhizal fungal population studies thus far, to enable direct comparison of the obtained *N*e values with values from plants and animals. The critical threshold in plant and animal conservation studies to avoid short-term extinction from inbreeding depression is often set at *N*e = 50, where a 1% increase in inbreeding per generation is assumed to be acceptable in terms of fitness over short time periods (Franklin, 1980). The estimated *N*e for *R. togasawarius* was below this threshold in four of the five populations and near the threshold in the remaining population, suggesting that inbreeding has been accumulating at alarming rates.

Accordingly, the inbreeding coefficient (*F*IS) was very high in four of the five populations and reached 0.120 in the KW population, supporting our Hypothesis 2. Although it is difficult to directly observe fungal fitness, some previous studies suggested the existence of inbreeding depression in fungi using artificially inbred strains under *in vitro* conditions (Eugenio & Anderson, 1968; Leslie & Raju, 1985; Xu, 1995; Malloure & James, 2013). For example, Malloure & James (2013) reported that mycelial growth decreased by approximately 15% in inbred strains of the bird’s nest fungus *Cyathus stercoreus* compared with outbred strains. Alternatively, the extinction risks of *R. togasawarius* can be inferred from the fitness data of plants and animals with similar inbreeding levels (although direct comparisons should be cautious due to the differences in their reproductive systems). For example, the observed *F*IS value in KW (0.120) was comparable to the value in the inbred population of *Silene alba* (0.125), which exhibited a 30% decrease in germination success (Richards et al., 1999). Similarly, inbreeding depression in Scottish red deer (*Cervus elaphus*) with a similar inbreeding coefficient value reduced first-year survival rates by half compared with outcrossed individuals (Walling et al., 2011). Indeed, *R. togasawarius* exhibits very infrequent ECM colonization and fruiting in mature *P. japonica* forests (Murata et al., 2013; Mujic et al., 2014; Orihara, 2019), potentially indicating inbreeding depression; the observed patterns contrast with the phylogenetically close but common species *R. vinicolor* and *R. vesiculosus* in North America, which abundantly colonize *P. menziesii* trees with frequent sporocarp production (Molina et al., 1999; Beiler et al., 2010).

What should also be noted here is that the observed inbreeding and loss of genetic diversity came from dormant spore banks, not from currently living fungal individuals; the number of living individuals is likely near zero, considering the absence of ECM roots in the resident tree roots (Murata et al., 2013). *Rhizopogon* spores remain dormant in the soil for extended periods, probably decades, awaiting disturbance to co-regenerate with their pioneer hosts (Baar et al., 1999; Kjøller & Bruns, 2003; Bruns et al., 2009; Nara, 2009; Shemesh et al., 2023). However, current conservation strategies for the host *P. japonica* involve ensuring that the remaining forests are undisturbed, prohibiting regeneration of the host (Yatoh, 1958; Yamanaka, 1975) and probably regeneration of *R. togasawarius*. Therefore, the observed inbreeding and genetic diversity loss in *R. togasawarius* spore banks may predate the large-scale deforestation that occurred in the 1950s. Because the recent human disturbance undoubtedly reduced the size of spore bank populations in proportion to the reduced forest area, the genetic diversity and inbreeding of *R. togasawarius* after the next regeneration opportunity, if it were to occur, would be much worse than observed here.

## Consistent genetic structures between *R. togasawarius* and its host populations

The phylogenetic topology of *R. togasawarius* was congruent with that of *P. japonica* populations, and their genetic differentiation (*F*ST) was significantly correlated. These results indicated that populations of the two species experienced the same fragmentation and divergence history, supporting our Hypothesis 3. To our knowledge, no previous studies have directly compared population genetic structures between ECM fungi and their hosts in the same analyses. Only Sheedy et al. (2015) mentioned that genetic differentiation among *Laccaria* sp. populations corresponded with the previously documented biogeography of the host *N. cunninghamii* (Sauquet et al., 2011).

Although we used SSRs for both *R. togasawarius* and *P. japonica*, the extent of genetic differentiation was considerably higher in *R. togasawarius* populations. One possible reason for the greater impact on fungal populations is the difference in gene flow mechanisms. Conifer trees, including *P*. *japonica*, release abundant wind-dispersed pollen, facilitating gene flow over long distances. For example, approximately 4.3% of *Pinus sylvestris* pollen travels more than 30 km (Robledo-Arnuncio & Gil, 2005). Indeed, genetic differentiation between *P*. *japonica* populations was not significant in the nearest population pair in each region. In contrast, similar to other *Rhizopogon* species, *R. togasawarius* spores are likely dispersed by mycophagous land animals, which migrate in a manner that is restricted by the small-scale geographic barriers separating fragmented *P*. *japonica* forests (Okada et al., 2022).

## Toward conservation of *R. togasawarius* and its implications for microbial conservation

A notable strength of our study is that all major populations of endangered *R. togasawarius* were included in population genetic analyses. To our knowledge, no previous studies on fungal populations adopted such an approach because of various technical difficulties involved in studying microbial populations across the entire distribution range, as mentioned above. In conservation genetics, it is important to identify where genetic diversity exists across the entire distribution range for the focal threatened species (Frankham et al., 2010). Due to the extremely restricted gene flow among populations and higher degrees of inbreeding within each population, most of the genetic diversity of *R. togasawarius* remains between and not within the populations. Therefore, when collecting culture strains for conservation, efforts should be focused on acquiring strains from different locations.

As cultured strains of ECM fungi often lose their infectivity during repeated subculturing (Marx & Daniel, 1976; Lalaymia et al., 2014), the collection and preservation of soil spore banks may provide better conservation results than the use of cultured strains. Such an approach may be feasible only for fungal species that produce dormant spores, such as *Rhizopogon* species. However, if successful, this method could preserve infective and genetically diverse spores until the expiration of their dormancy. The spore bank is analogous to plant seed banks, and *P. japonica* seeds have been collected for seed banks during fruiting years spaced several years apart (Isoda et al., 2014). Collecting soil spores during these seed collection efforts would allow the two species to be preserved simultaneously. As shown in this study, we could then reproduce their ECM relationship from the resulting spore banks, even under *ex situ* conditions.

Soil spore banks can also be collected from extinct patches of *P. japonica* forest, where *P. japonica* trees were recorded before the 1950s (Yamanaka, 1975) but have been completely replaced by conifer plantations. Based on the significant genetic differentiation observed among the nearest extant populations in this study, *R. togasawarius* spores in these extinct *P. japonica* forest patches are likely genetically unique, differing from the remaining populations. Reviving these microbes from soil spore banks would have a broad impact on microbial conservation. Determining how long the spores can survive is essential to the use of soil spore banks for preservation measures. A spore burial experiment demonstrated that spores of North American *Rhizopogon* species retained infectivity for at least 15 years, although their infectivity decreased after 10 years (Shemesh et al., 2023). While we are uncertain whether *R. togasawarius* spores remain viable for more than 60 years in conifer plantation soil, such extinct stands provide an interesting opportunity to examine the longevity of spore banks over decades and their genetic structures, using the reference dataset developed in this study.

The population genetic data obtained in this study can be used to identify conservation units (CUs), which is a crucial step for conserving endangered species (Ryder, 1986; Fraser & Bernatchez, 2001; Hoelzel, 2023). Our data indicate that individual *R. togasawarius* populations have been reproductively isolated for thousands of generations without gene flow, supporting their treatment as individual CUs for *in situ* conservation. Although phenotypic and life history data are sometimes employed in defining CUs for plants and animals (Ryder, 1986; Lehnert et al., 2023; Molinari, 2023), such data are often unobtainable for fungal species (Kraemer & Boynton, 2017), particularly *R. togasawarius*, which exists exclusively as dormant spores in soil (Murata et al., 2017). Therefore, introducing genetically different individuals into *R. togasawarius* populations without solid evidence against local adaptation requires caution, as these individuals may carry genes that impair local environmental adaptation.

For *in situ* conservation of ECM fungi, interacting species must be considered (Lofgren & Stajich, 2021). Due to the rare fruiting of *R. togasawarius*, specific dispersers of this fungus are unlikely to exist; instead, its spores are likely dispersed by common animals such as deer and rodents (Luoma et al., 2003; Vašutová et al., 2019; Elliott et al., 2022). This possibility is supported by the absence of any animal species described as feeding solely on *Rhizopogon* sporocarps (Elliott et al., 2022). Thus, the management of such animals, which have large populations in our study areas (Miyao et al., 1965; Kaneshiro & Yachimori, 2022; Takagi et al., 2023), may not be necessary for *R. togasawarius* conservation. Alternately, compatible hosts that provide carbohydrates for ECM fungi are an important research focus. As *R. togasawarius* is exclusively compatible with *P. japonica* and can regenerate with its seedlings after disturbance (Murata et al., 2017), promoting *in situ* regeneration of *P. japonica* seedlings is essential. One practical and feasible strategy is to create gaps at the periphery of remaining forest patches, as *P. japonica* is a light-demanding species. For example, plantations established in the 1950s could be clear-cut in areas where *R. togasawarius* spores have been confirmed in the soil (up to 300 m from the remaining *P. japonica* forest; Okada et al., 2022). After clear-cutting, light-demanding *P. japonica* seedlings and their specific symbiont *R. togasawarius* could regenerate simultaneously, creating new spore banks for future generations before depleting existing spore banks. Through this *in situ* approach, the remaining forest patches could be expanded to occupy their historical distribution ranges prior to human disturbance, thereby reducing genetic decline in future generations at each site and mitigating the extinction risk associated with stochastic disasters (e.g., forest fires and landslides).

The high inbreeding rates, decreased genetic diversity, and small effective population sizes observed in all *R. togasawarius* populations further indicate a potentially serious risk of extinction through inbreeding depression, as reported in numerous plants and animals with small, fragmented populations (Frankham et al., 2017). Whether the fitness of these inbred microbial populations has already declined remains unknown, as the life history traits of this species are difficult to observe. However, reduced fitness (e.g., lower seed germination and more empty seeds compared to the congeneric *P. menziesii*) is becoming evident in its specific host, *P. japonica* (Iwaizumi et al., personal communication), which exhibits weakly significantly correlated genetic differentiation with *R. togasawarius*. To examine the reduced fitness of inbred *R. togasawarius* individuals, producing outbred individuals and comparing their life history traits (e.g., mycelial growth, ECM colonization, host growth, and sporocarp production) with inbred individuals under *ex situ* conditions could be a useful first step. We have already confirmed that outbred strains can be readily obtained by mixing soil spore banks from various sites and employing the bioassay described in this study. If outbred individuals outperform inbred individuals in these traits, it may be necessary to consider genetic rescue through the introduction of genetically diverse individuals from other populations to reduce inbreeding depression and restore genetic diversity. This approach has been successful in the conservation of many plant and animal species (Frankham, 2015; Ralls et al., 2020). When establishing an *ex situ* *P. japonica* conservation plantation, it is possible to use soil spore banks of mixed origin to encourage outbred *R. togasawarius* to form ECM relationships with the transplanted seedlings. Soil spore banks of *R. togasawarius*, and likely also those of other spore bank-forming microbe species, can be transferred easily among populations or to *ex situ* sites, enabling their use in diverse conservation measures.

No conservation activities have been undertaken for *R. togasawarius,* partly because this recently described species (Mujic et al., 2014) has yet to be listed on Japan’s Red List (Ministry of the Environment of Japan, 2019). Based on scientific data showing its strict specificity to an endangered host (Murata et al., 2017), undetectably rare ECM colonization of resident tree roots (Murata et al., 2013), distribution ranges of spore banks (Okada et al., 2022), and discrete genetic structures with limited gene flow over thousands of generations (this study), we hope that this species will be included in the next update of Japan’s Red List, similar to its listing on the IUCN Red List (IUCN, 2023). Although our data provide valuable information for assessing the extinction risk of *R. togasawarius* for the Red Lists, including determining its CUs, identifying populations for prioritized conservation efforts, and proposing potential conservation measures, further research is needed prior to the implementation of any such conservation measures. In addition, conservation efforts should be broadened to include other potentially endangered fungal species using the approaches described in this study. In the IUCN Red List (IUCN, 2023), nearly 50 species of *Pinus* and *Pseudotsuga* are categorized as threatened or near threatened. Given that these trees may be associated with specific *Rhizopogon* fungi similar to *R. togasawarius* (Mujic et al., 2014; Murata et al., 2017), many other endangered *Rhizopogon* species could be identified from these threatened hosts. For example, *R. yakushimensis* is specific to endangered *Pinus amamiana* (Sugiyama et al., 2018; Murata et al., 2017) and is now included in the IUCN Red List (IUCN, 2023). Similarly, the conservation of other hypogeous ECM or saprotrophic fungal species associated with specific host species may require additional attention. Overall, the data obtained in this initial conservation genetic study of ECM microbes may stimulate and broaden conservation efforts for these organisms, which play various essential roles in ecosystems but have been overlooked in conservation research and activities compared to plants and animals.

**Acknowledgements**

We express great gratitude to Akira Ishikawa and the members of the laboratory for their helpful comments about bioinformatics on this research. We are grateful to Dr. Yosuke Matsuda, Dr. Atsushi Sakai, Dr. Masakazu Iwaizumi, Dr. Keita Okada for field sampling. We also offer our thanks to the anonymous reviewers for their valuable suggestions. This work was supported by JSPS KAKENHI Grant (KAKENHI 15H02449, 18H03955, 23H00340).

**References**

Abe, H., Tabuchi, A., Okuda, Y., & Matsumoto, T. (2018). Genetic structure analyses of ectomycorrhizal fungus, *Rhizopogon roseolus* by SSR markers in three spatial scales. *Journal of the Japanese Forest Society,***100**, 8-14. <https://doi.org/10.4005/jjfs.100.8> (in Japanese).

Abe, H., Tabuchi, A., Okuda, Y., Matsumoto, T., & Nara, K. (2017). Population genetics and fine-scale genetic structure of *Rhizopogon roseolus* in the Tottori sand dune *Mycoscience,***58**, 14-22. <https://doi.org/10.1016/j.myc.2016.07.009>

Aguilar, R., Cristóbal-Pérez, E. J., Balvino-Olvera, F. J., Aguilar-Aguilar, M. J., Aguirre-Acosta, N., Ashworth, L., Lobo, J. A., Martén-Rodríguez, S., Fuchs, E. J., Sanchez-Montoya, G., Bernardello, G., & Quesada, M. (2019). Habitat fragmentation reduces plant progeny quality: a global synthesis. *Ecology Letters,***22**, 1163-1173. <https://doi.org/10.1111/ele.13272>

Amarasinghe, V., & Carlson, J. E. (2002). The development of microsatellite DNA markers for genetic analysis in Douglas-fir. *Canadian Journal of Forest Research,***32**, 1904-1915. <https://doi.org/10.1139/x02-110>

Arnaud-Haond, S., & Belkhir, K. (2007). GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes,***7**, 15-17. <https://doi.org/10.1111/j.1471-8286.2006.01522.x>

Baar, J., Horton, T. R., Kretzer A. M., & Bruns, T. D. (1999). Mycorrhizal colonization of *Pinus muricata* from resistant propagules after a stand-replacing wildfire. *New Phytologist,***143**, 409-418. <https://doi.org/10.1046/j.1469-8137.1999.00452.x>

Beiler, K. J., Durall, D. M., Simard, S. W., Maxwell, S. A., & Kretzer, A. M. (2010). Architecture of the wood-wide web: *Rhizopogon* spp. genets link multiple Douglas-fir cohorts. *New Phytologist,***185**, 543-553. <https://doi.org/10.1111/j.1469-8137.2009.03069.x>

Bialozyt, R., Ziegenhagen, B., & Petit, R. J. (2006). Contrasting effects of long distance seed dispersal on genetic diversity during range expansion. *Journal of Evolutionary Biology,***19**, 12-20. <https://doi.org/10.1111/j.1420-9101.2005.00995.x>

Blacket, M.J., Robin, C., Good, R.T., Lee, S.F., & Miller, A. D. (2012). Universal primers for fluorescent labelling of PCR fragments—an efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources,***12**, 456-463. <https://doi.org/10.1111/j.1755-0998.2011.03104.x>

Bohrer, G., Nathan, R., & Volis, S. (2005). Effects of long-distance dispersal for metapopulation survival and genetic structure at ecological time and spatial scales. *Journal of Ecology,***93**, 1029-1040. <https://doi.org/10.1111/j.1365-2745.2005.01048.x>

Borgmann-Winter, B., Stephens, R. B., Anthony, M. A., Frey, S. D., D'Amato, A. W., & Rowe, R. J. (2023). Wind and small mammals are complementary fungal dispersers. *Ecology,***104**, e4039. <https://doi.org/10.1002/ecy.4039>

Bruns, T. D., Peay, K. G., Boynton, P. J., Grubisha, L. C., Hynson, N. A., Nguyen, N. H., & Rosenstock, N. P. (2009). Inoculum potential of *Rhizopogon* spores increases with time over the first 4 yr of a 99-yr spore burial experiment. *New Phytologist,***181**, 463-470. <https://doi.org/10.1111/j.1469-8137.2008.02652.x>

Buscardo, E., Rodríguez-Echeverría, S., Martín, M. P., De Angelis, P., Pereira, J. S., & Freitas, H. (2010). Impact of wildfire return interval on the ectomycorrhizal resistant propagules communities of a Mediterranean open forest. *Fungal Biology,***114**, 628-636. <https://doi.org/10.1016/j.funbio.2010.05.004>

Charlesworth, D., & Willis, J. H. (2009). The genetics of inbreeding depression. *Nature Reviews Genetics,***10**, 783-796. <http://doi.org/10.1038/nrg2664>

Collin, F., Durif, G., Raynal, L., Lombaert, E., Gautier, M., Vitalis, R., Marin, J., & Estoup, A. (2021). Extending approximate Bayesian computation with supervised machine learning to infer demographic history from genetic polymorphisms using DIYABC Random Forest. *Molecular Ecology Resources,***21**, 2598-2613. <https://doi.org/10.1111/1755-0998.13413>

Cornuet, J. M., & Luikart, G. (1996). Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics,***144**, 2001-2014. <http://doi.org/10.1093/genetics/144.4.2001>

Crnokrak, P., & Barrett, S. C. H. (2002). Perspective: purging the genetic load: a review of the experimental evidence. *Evolution,***56**, 2347-2358. <https://doi.org/10.1111/j.0014-3820.2002.tb00160.x>

Dahlberg, A., & Mueller, G. M. (2011). Applying IUCN red-listing criteria for assessing and reporting on the conservation status of fungal species. *Fungal Ecology,***4**, 147-162. <https://doi.org/10.1016/j.funeco.2010.11.001>

Do, C., Waples, R. S., Peel, D., Macbeth, G. M., Tillett, B. J., & Ovenden, J. R. (2014). NE ESTIMATOR v2: re-implementation of software for the estimation of contemporary effective population size (*N*e) from genetic data. *Molecular Ecology Resources,***14**, 209-214. <https://doi.org/10.1111/1755-0998.12157>

Douhan, G. W., Vincenot, L., Gryta, H., & Selosse, M. (2011). Population genetics of ectomycorrhizal fungi: from current knowledge to emerging directions. *Fungal Biology,***115**, 569-597. <https://doi.org/10.1016/j.funbio.2011.03.005>

Dunham, S. M., Mujic, A. B., Spatafora, J. W., & Kretzer, A. M. (2013). Within-population genetic structure differs between two sympatric sister-species of ectomycorrhizal fungi, *Rhizopogon vinicolor* and *R. vesiculosus*. *Mycologia,***105**, 814-826. <https://doi.org/10.3852/12-265>

Dunham, S. M., O’Dell, T. E., & Molina, R. (2006). Spatial analysis of within-population microsatellite variability reveals restricted gene flow in the Pacific golden chanterelle (*Cantharellus formosus*). *Mycologia,***98**, 250-259. <https://doi.org/10.1080/15572536.2006.11832697>

Dutech, C., Enjalbert, J., Fournier, E., Delmotte, F., Barrès, B., Carlier, J., Tharreau, D., & Giraud, T. (2007). Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology,***44**, 933-949. <https://doi.org/10.1016/j.fgb.2007.05.003>

Ellegren, H. (2000). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics,***16**, 551-558. <https://doi.org/10.1016/S0168-9525(00)02139-9>

Elliott, T. F., Truong, C., Jackson, S. M., Zúñiga, C. L., Trappe, J. M., & Vernes, K. (2022). Mammalian Mycophagy: a Global Review of Ecosystem Interactions Between Mammals and Fungi. *Fungal Systematics and Evolution,***9**, 99-159. <https://doi.org/10.3114/fuse.2022.09.07>

Eugenio, C. P., & Anderson, N. A. (1968). The genetics and cultivation of *Pleurotus ostreatus*. *Mycologia,***60**, 627-634. <https://doi.org/10.1080/00275514.1968.12018612>

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular ecology,***14**, 2611-2620. <https://doi.org/10.1111/j.1365-294X.2005.02553.x>

Fahrig, L. (2003). Effects of habitat fragmentation on biodiversity. *Annual Review of Ecology, Evolution, and Systematics,***34**, 487-515. <https://doi.org/10.1146/annurev.ecolsys.34.011802.132419>

Francis, R. M. (2017). POPHELPER: an R package and web app to analyse and visualize population structure. *Molecular Ecology Resources,***17**, 27-32. <https://doi.org/10.1111/1755-0998.12509>

Frankham, R. (2015). Genetic rescue of small inbred populations: meta-analysis reveals large and consistent benefits of gene flow. *Molecular ecology,***24**, 2610-2618.　 <https://doi.org/10.1111/mec.13139>

Frankham, R., Ballou, J. D., Ralls, K., Eldridge, M., Dudash, M. R., Fenster, C. B., Lacy, R. C., & Sunnucks, P. (2017). *Genetic Management of Fragmented Animal and Plant Populations*. Oxford Academic. <https://doi.org/10.1093/oso/9780198783398.001.0001>

Frankham, R., Ballou, J. D., & Briscoe, D. A. (2010). *Introduction to Conservation Genetics, 2nd edn.* Cambridge University Press.

Franklin, I. R. (1980). Evolutionary change in small populations. In M. E. Soulé & B. A. Wilcox (Eds.), *Conservation biology: An evolutionary-ecological perspective* (pp. 135–149). Sinauer Associates.

Fraser, D. J., & Bernatchez, L. (2001). Adaptive evolutionary conservation: Towards a unified concept for defining conservation units. *Molecular Ecology*, **10**, 2741-2752.

Glassman, S. I., Levine, C. R., DiRocco, A. M., Battles, J. J., & Bruns, T. D. (2016). Ectomycorrhizal fungal spore bank recovery after a severe forest fire: some like it hot. *The ISME Journal,***10**, 1228-1239. <https://doi.org/10.1038/ismej.2015.182>

Goudet, J. (1995). FSTAT (Version 1.2): A Computer Program to Calculate *F*-Statistics. *Journal of Heredity,***86**, 485-486.　<https://doi.org/10.1093/oxfordjournals.jhered.a111627>

Grubisha, L. C., Bergemann, S. E., & Bruns, T. D. (2007). Host islands within the California Northern Channel Islands create fine-scale genetic structure in two sympatric species of the symbiotic ectomycorrhizal fungus *Rhizopogon*. *Molecular ecology,***16**, 1811-1822. <https://doi.org/10.1111/j.1365-294X.2007.03264.x>

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Léger, P., Lepoittevin, C., Malausa, T., Revardel, E., Salin, F., & Petit, R. J. (2011). Current trends in microsatellite genotyping. *Molecular Ecology Resources,***11**, 591-611. <https://doi.org/10.1111/j.1755-0998.2011.03014.x>

Hedrick, P. W. (2005). A standardized genetic differentiation measure. *Evolution,***59**, 1633-1638.　<https://doi.org/10.1111/j.0014-3820.2005.tb01814.x>

Hedrick, P. W., & Garcia-Dorado, A. (2016). Understanding inbreeding depression, purging, and genetic rescue. *Trends in Ecology & Evolution,***31**, 940-952. <https://doi.org/10.1016/j.tree.2016.09.005>

Hitchcock, C. J., Chambers, S. M., & Cairney, J. W. G. (2011). Genetic population structure of the ectomycorrhizal fungus *Pisolithus microcarpus* suggests high gene flow in south-eastern Australia. *Mycorrhiza,***21**, 131-137. <https://doi.org/10.1007/s00572-010-0317-3>

Hoelzel, A. R. (2023). Where to now with the evolutionarily significant unit? *Trends in Ecology & Evolution*, **38**, 1134-1142. <https://doi.org/10.1016/j.tree.2023.07.005>

Hubisz, M. J., Falush, D., Stephens, M., & Pritchard, J. K. (2009). Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources,***9**, 1322-1332. <https://doi.org/10.1111/j.1755-0998.2009.02591.x>

Huson, D. H. (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics,***14**, 68-73. <https://doi.org/10.1093/bioinformatics/14.1.68>

Huson, D. H., & Bryant, D. (2005). Application of Phylogenetic Networks in Evolutionary Studies. *Molecular biology and evolution,***23**, 254-267. <https://doi.org/10.1093/molbev/msj030>

Ishikawa, A., & Nara, K. (2023). Primary succession of ectomycorrhizal fungi associated with *Alnus sieboldiana* on Izu-Oshima Island, Japan. *Mycorrhiza,***33**, 187-197. <https://doi.org/10.1007/s00572-023-01112-w>

Isoda, K., Sasajima, Y., & Iwaizumi, M. (2014). Conservation of endangered tree species in Kinki, Chugoku, and Shikoku area. *Tree and Forest Health*, **18**, 57-61. <https://doi.org/10.18938/treeforesthealth.18.3_57> (in Japanese).

IUCN. (2023). The IUCN Red List of Threatened Species. Version 2023-1. Retrieved from https://www.iucnredlist.org. Accessed on 29 March 2014.

Jump, A. S., & Peñuelas, J. (2006). Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. *Proceedings of the National Academy of Sciences,***103**, 8096-8100. <https://doi.org/10.1073/pnas.0510127103>

Kaneshiro, Y., Yachimori, S. (2022). List of mammals from Kochi Prefecture. *Bulletin of Shikoku Institute of Natural History*, **15**, 70-73. <https://doi.org/10.32250/sinh.15.0_70> (in Japanese).

Kipfer, T., Moser, B., Egli, S., Wohlgemuth, T., & Ghazoul, J. (2011). Ectomycorrhiza succession patterns in *Pinus sylvestris* forests after stand-replacing fire in the Central Alps. *Oecologia,***167**, 219-228. <https://doi.org/10.1007/s00442-011-1981-5>

Kjøller, R., & Bruns, T. D. (2003). *Rhizopogon* spore bank communities within and among California pine forests. *Mycologia,***95**, 603-613. <https://doi.org/10.1080/15572536.2004.11833064>

Kraemer, S. A., & Boynton, P. J. (2017). Evidence for microbial local adaptation in nature. *Molecular Ecology*, **26**, 1860-1876. <https://doi.org/10.1111/mec.13958>

Kretzer, A. M., Dunham, S., Molina, R., & Spatafora, J. W. (2005). Patterns of vegetative growth and gene flow in *Rhizopogon vinicolor* and *R. vesiculosus* (Boletales, Basidiomycota). *Molecular ecology,***14**, 2259-2268. <https://doi.org/10.1111/j.1365-294X.2005.02547.x>

Kretzer, A. M., Dunham, S., Molina, R., & Spatafora, J. W. (2004). Microsatellite markers reveal the below ground distribution of genets in two species of *Rhizopogon* forming tuberculate ectomycorrhizas on Douglas fir. *New Phytologist,***161**, 313-320. <https://doi.org/10.1046/j.1469-8137.2003.00915.x>

Kretzer, A. M., Molina, R., & Spatafora, J. W. (2000). Microsatellite markers for the ectomycorrhizal basidiomycete *Rhizopogon vinicolor*. *Molecular ecology,***9**, 1190-1191. <https://doi.org/10.1046/j.1365-294x.2000.00954-12.x>

Labbé, F., Fontaine, M. C., Robin, C., & Dutech, C. (2017). Genetic signatures of variation in population size in a native fungal pathogen after the recent massive plantation of its host tree. *Heredity,***119**, 402-410. <https://doi.org/10.1038/hdy.2017.58>

Lalaymia, I., Cranenbrouck, S., & Declerck, S. (2014). Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi. *Mycorrhiza*, **24**, 323-337. <https://doi.org/10.1007/s00572-013-0541-8>

Lehnert, S. J., Bradbury, I. R., Wringe, B. F., Van Wyngaarden, M., & Bentzen, P. (2023). Multifaceted framework for defining conservation units: An example from Atlantic salmon (*Salmo salar*) in Canada. *Evolutionary Applications*, **16**, 1568-1585. <https://doi.org/10.1111/eva.13587>

Leslie, J. F., & Raju, N. B. (1985). Recessive Mutations from natural populations of *Neurospora crassa* that are expressed in the sexual diplophase. *Genetics,***111**, 759-777. <https://doi.org/10.1093/genetics/111.4.759>

Lian, C., & Hogetsu, T. (2002). Development of microsatellite markers in black locust (*Robinia pseudoacacia*) using a dual-supression-PCR technique. *Molecular Ecology Notes,***2**, 211-213. <https://doi.org/10.1046/j.1471-8286.2002.00213.x-i2>

Lofgren, L. A., & Stajich, J. E. (2021). Fungal biodiversity and conservation mycology in light of new technology, big data, and changing attitudes. *Current Biology*, **31**, 1312-1325. <https://doi.org/10.1016/j.cub.2021.06.083>

Lowe, A.J., Boshier, D., Ward, M., Bacles, C.F.E., & Navarro, C. (2005). Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. *Heredity,***95**, 255-273. <https://doi.org/10.1038/sj.hdy.6800725>

Luoma, D.L., Trappe, J.M., Claridge, A.W., Jacobs, K.M., & Cazares, E. (2003). Relationships among fungi and small mammals in forested ecosystems. In Zabel, C., & Anthony, R. G. (Eds.), *Mammal Community Dynamics in Western Coniferous Forests: Management and Conservation* (pp. 343–373). Cambridge University Press.

Malloure, B.D., & James, T.Y. (2013). Inbreeding depression in urban environments of the bird’s nest fungus *Cyathus stercoreus* (Nidulariaceae: Basidiomycota). *Heredity,***110**, 355-362. <https://doi.org/10.1038/hdy.2012.95>

Marx, D. H., & Daniel, W. J. (1976). Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. *Canadian Journal of Microbiology*, **22**, 338-341. <https://doi.org/10.1139/m76-051>

May, T. W., Cooper, J. A., Dahlberg, A., Furci, G., Minter, D. W., Mueller, G. M., Pouliot, A., & Yang, Z. (2019). Recognition of the discipline of conservation mycology. *Conservation Biology*, **33**, 733-736. <https://doi.org/10.1111/cobi.13228>

Ministry of the Environment of Japan. (2019). Ministry of the environment red list 2019. Retrieved from https://www.env.go.jp/content/900515981.pdf. Accessed 4 June 2024

Miyao, T., Akahane, H., Sakai, A., & Yanagidaira, Y. (1965). Small mammals of the mountainous part in the Kii Peninsula. *The Journal of the Mammalogical Society of Japan*, **2**, 120-123 (in Japanese).

Mohanta, T.K., & Bae, H. (2015). The diversity of fungal genome. *Biological Procedures Online,***17**, 8. <https://doi.org/10.1186/s12575-015-0020-z>

Molina, R., Trappe, J.M., Grubisha, L.C., & Spatafora, J.W. (1999). Rhizopogon. In Cairney, J. W. G., & Chambers, S. M. (Eds.), *Ectomycorrhizal Fungi Key Genera in Profile* (pp. 129–161). Springer.

Molinari, J. (2023). A bare-bones scheme to choose between the species, subspecies, and 'evolutionarily significant unit' categories in taxonomy and conservation. *Journal for Nature Conservation*, **72**, 126335. <https://doi.org/10.1016/j.jnc.2023.126335>

Mori, I., & Kumazaki, M. (1990). *Sensasu ni Miru Nihon no Ringyou* (Census of Japanese forestry). Zenkoku-Nourin-Toukei-Kyoukai-Rengoukai (in Japanese).

Mujic, A. B., Hosaka, K., & Spatafora, J. W. (2014). *Rhizopogon togasawariana* sp. nov., the first report of *Rhizopogon* associated with an Asian species of *Pseudotsuga*. *Mycologia,***106**, 105-112. <https://doi.org/10.3852/13-055>

Mujic, A. B., Huang, B., Chen, M., Wang, P., Gernandt, D. S., Hosaka, K., & Spatafora, J. W. (2019). Out of western North America: Evolution of the *Rhizopogon*-*Pseudotsuga* symbiosis inferred by genome-scale sequence typing. *Fungal Ecology,***39**, 12-25. <https://doi.org/10.1016/j.funeco.2018.10.006>

Murata, M., Kinoshita, A., & Nara, K. (2013). Revisiting the host effect on ectomycorrhizal fungal communities: implications from host–fungal associations in relict *Pseudotsuga japonica* forests. *Mycorrhiza,***23**, 641-653. <https://doi.org/10.1007/s00572-013-0504-0>

Murata, M., Nagata, Y., & Nara, K. (2017). Soil spore banks of ectomycorrhizal fungi in endangered Japanese Douglas-fir forests. *Ecological Research,***32**, 469-479. <https://doi.org/10.1007/s11284-017-1456-1>

Nakamura, J., & Katto, J. (1953). Palynological studies of the Quaternary formations. 2. Coastal terrace deposits at Oono and Sawanohira, Kochi Prefecture, Shikoku. *Bulletin of the Society of Plant Ecology,***3**, 108–111 (in Japanese).

Nara, K. (2009). Spores of ectomycorrhizal fungi: ecological strategies for germination and dormancy. *New Phytologist,***181**, 245-248. <https://doi.org/10.1111/j.1469-8137.2008.02691.x>

Nara, K. (2006a). Ectomycorrhizal networks and seedling establishment during early primary succession. *New Phytologist,***169**, 169-178. <https://doi.org/10.1111/j.1469-8137.2005.01545.x>

Nara, K. (2006b). Pioneer dwarf willow may facilitate tree succession by providing late colonizers with compatible ectomycorrhizal fungi in a primary successional volcanic desert. *New Phytologist,***171**, 187-198. <https://doi.org/10.1111/j.1469-8137.2006.01744.x>

Nara, K., Nakaya, H., Wu, B., Zhou, Z., & Hogetsu, T. (2003). Underground primary succession of ectomycorrhizal fungi in a volcanic desert on Mount Fuji. *New Phytologist,***159**, 743-756. <https://doi.org/10.1046/j.1469-8137.2003.00844.x>

Navascués, M., Leblois, R., & Burgarella, C. (2017). Demographic inference through approximate-Bayesian-computation skyline plots. *PeerJ*, **5**, e3530. <https://doi.org/10.7717/peerj.3530>

Nei, M., Tajima, F., & Tateno, Y. (1983). Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution,***19**, 153-170. <https://doi.org/10.1007/BF02300753>

Okada, K. H., Abe, H., Matsuda, Y., & Nara, K. (2022). Spatial distribution of spore banks of ectomycorrhizal fungus, *Rhizopogon togasawarius*, at *Pseudotsuga japonica* forest boundaries. *Journal of Forest Research,***27**, 308-314. <https://doi.org/10.1080/13416979.2021.2023386>

Okada, K. H., & Matsuda, Y. (2022). Soil spore bank communities of ectomycorrhizal fungi in *Pseudotsuga japonica* forests and neighboring plantations. *Mycorrhiza,***32**, 83-93. <https://doi.org/10.1007/s00572-021-01065-y>

Ooi, N. (2016). Vegetation history of Japan since the last glacial based on palynological data. *Japanese journal of historical botany,***25**, 1-101. <https://doi.org/10.34596/hisbot.25.1-2_1>

Orihara, T. (2019). First discovery of *Rhizopogon togasawariana* (Rhizopogonaceae, Boletales) from Wakayama Prefecture, Japan. *Truffology,***2**, 18-19 (in Japanese).

Peakall, R., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,***6**, 288-295. <https://doi.org/10.1111/j.1471-8286.2005.01155.x>

Peay, K. G., Garbelotto, M., & Bruns, T. D. (2009). Spore heat resistance plays an important role in disturbance-mediated assemblage shift of ectomycorrhizal fungi colonizing *Pinus muricata* seedlings. *Journal of Ecology,***97**, 537-547. <https://doi.org/10.1111/j.1365-2745.2009.01489.x>

Pildain, M. B., Marchelli, P., Azpilicueta, M. M., Starik, C., & Barroetaveña, C. (2021). Understanding introduction history: Genetic structure and diversity of the edible ectomycorrhizal fungus, *Suillus luteus*, in Patagonia (Argentina). *Mycologia,***113**, 715-724. <https://doi.org/10.1080/00275514.2021.1909449>

Piry, S., Luikart, G., & Cornuet, J. (1999). BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. *Journal of Heredity,***90**, 502-503. <https://doi.org/10.1093/jhered/90.4.502>

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945-959. <https://doi.org/10.1093/genetics/155.2.945>

Queirós, J., Godinho, R., Lopes, S., Gortazar, C., de la Fuente, J., & Alves, P. C. (2015). Effect of microsatellite selection on individual and population genetic inferences: an empirical study using cross-specific and species-specific amplifications. *Molecular Ecology Resources,***15**, 747-760. <https://doi.org/10.1111/1755-0998.12349>

R Core Team. (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Retrieved from <https://www.R-project.org/>

Ralls, K., Sunnucks, P., Lacy, R. C., & Frankham, R. (2020). Genetic rescue: A critique of the evidence supports maximizing genetic diversity rather than minimizing the introduction of putatively harmful genetic variation. *Biological Conservation*, **251**, 108784. <https://doi.org/10.1016/j.biocon.2020.108784>

Richards, C. M., Church, S., & McCauley, D. E. (1999). The influence of population size and isolation on gene flow by pollen in *Silene alba*. *Evolution*, **53**, 63-73. <https://doi.org/10.1111/j.1558-5646.1999.tb05333.x>

Rivera, Y., Burchhardt, K. M., & Kretzer, A. M. (2014). Little to no genetic structure in the ectomycorrhizal basidiomycete *Suillus spraguei* (Syn. *S. pictus*) across parts of the northeastern USA. *Mycorrhiza,***24**, 227-232. <https://doi.org/10.1007/s00572-013-0524-9>

Robledo-Arnuncio, J., & Gil, L. (2005). Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Heredity,***94**, 13-22. <https://doi.org/10.1038/sj.hdy.6800542>

Ryder, O. A. (1986). Species conservation and systematics: The dilemma of subspecies. *Trends in Ecology & Evolution*, **1**, 9-10. <https://doi.org/10.1016/0169-5347(86)90059-5>

Sauquet, H., Ho, S. Y. W., Gandolfo, M. A., Jordan, G. J., Wilf, P., Cantrill, D. J., Bayly, M. J., Bromham, L., Brown, G. K., Carpenter, R. J., Lee, D. M., Murphy, D. J., Sniderman, J. M. K., & Udovicic, F. (2012). Testing the impact of calibration on molecular divergence times using a fossil-rich group: The case of *Nothofagus* (Fagales). *Systematic Biology*, **61**(2), 289-313. <https://doi.org/10.1093/sysbio/syr116>

Schlaepfer, D. R., Braschler, B., Rusterholz, H., & Baur, B. (2018). Genetic effects of anthropogenic habitat fragmentation on remnant animal and plant populations: a meta-analysis. *Ecosphere,***9**, e02488. <https://doi.org/10.1002/ecs2.2488>

Sheedy, E. M., Van de Wouw, A. P., Howlett, B. J., & May, T. W. (2015). Population genetic structure of the ectomycorrhizal fungus *Laccaria* sp. A resembles that of its host tree *Nothofagus cunninghamii*. *Fungal Ecology,***13**, 23-32. <https://doi.org/10.1016/j.funeco.2014.08.005>

Shemesh, H., Bruns, T. D., Peay, K. G., Kennedy, P. G., & Nguyen, N. H. (2023). Changing balance between dormancy and mortality determines the trajectory of ectomycorrhizal fungal spore longevity over a 15-yr burial experiment. *New Phytologist,***238**, 11-15. <https://doi.org/10.1111/nph.18677>

Shimakura, M. (1969). Plant Micro fossils from Marine Pleistocene Sediments of Kii Peninsula, Japan. *Bulletin of Nara University of Education,***17**(2), 75-88.

Slavov, G. T., Howe, G. T., Yakovlev, I., et al. (2004). Highly variable SSR markers in Douglas-fir: Mendelian inheritance and map locations. *Theoretical and Applied Genetics,***108**, 873-880. <https://doi.org/10.1007/s00122-003-1490-y>

Smith, S. E., & Read, D. (2008). *Mycorrhizal symbiosis* (3rd edn.). Academic Press.

Sugiyama, Y., Murata, M., & Nara, K. (2018). A new *Rhizopogon* species associated with *Pinus amamiana* in Japan. *Mycoscience*, **59**, 176-180. <https://doi.org/10.1016/j.myc.2017.10.001>

Takagi, T., Murakami, R., Takano, A., Torii, H., Kaneko, S., & Tamate, H. B. (2023). A historic religious sanctuary may have preserved ancestral genetics of Japanese sika deer (*Cervus nippon*). *Journal of Mammalogy*, **104**, 303-315. <https://doi.org/10.1093/jmammal/gyac120>

Takezaki, N., Nei, M., & Tamura, K. (2009). POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with Windows interface. *Molecular biology and evolution,***27**, 747-752. <https://doi.org/10.1093/molbev/msp312>

Tamaki, S., Isoda, K., Takahashi, M., Yamada, H., & Yamashita, Y. (2018). Genetic structure and diversity in relation to the recently reduced population size of the rare conifer, *Pseudotsuga japonica*, endemic to Japan. *Conservation Genetics,***19**, 1243-1255. <https://doi.org/10.1007/s10592-018-1092-5>

Tsykun, T., Rellstab, C., Dutech, C., Sipos, G., & Prospero, S. (2017). Comparative assessment of SSR and SNP markers for inferring the population genetic structure of the common fungus Armillaria cepistipes. *Heredity,***119**, 371-380. <https://doi.org/10.1038/hdy.2017.48>

Väli, Ü., Einarsson, A., Waits, L., & Ellegren, H. (2008). To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? *Molecular ecology,***17**, 3808-3817. <https://doi.org/10.1111/j.1365-294X.2008.03876.x>

Vašutová, M., Mleczko, P., López-García, A., Maček, I., Boros, G., Ševčík, J., Fujii, S., Hackenberger, D., Tuf, I. H., Hornung, E., Páll-Gergely, B., & Kjøller, R. (2019). Taxi drivers: the role of animals in transporting mycorrhizal fungi. *Mycorrhiza,***29**, 413-434. <https://doi.org/10.1007/s00572-019-00906-1>

Vincenot, L., Nara, K., Sthultz, C., Labbé, J., Dubois, M, Tedersoo, L., Martin, F., & Selosse, M. (2012). Extensive gene flow over Europe and possible speciation over Eurasia in the ectomycorrhizal basidiomycete *Laccaria amethystina* complex. *Molecular ecology,***21**, 281-299. <https://doi.org/10.1111/j.1365-294X.2011.05392.x>

Walling, C. A., Nussey, D. H., Morris, A., Clutton-Brock, T., Kruuk, L. E. B., & Pemberton, J. M. (2011). Inbreeding depression in red deer calves. *BMC Evolutionary Biology,***11**, 318. <https://doi.org/10.1186/1471-2148-11-318>

Waples, R. S., & Do, C. (2010). Linkage disequilibrium estimates of contemporary Ne using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. *Evolutionary Applications,***3**, 244-262. <https://doi.org/10.1111/j.1752-4571.2009.00104.x>

Willoughby, J. R., Sundaram, M., Wijayawardena, B. K., Kimble, S. J. A., Ji, Y., Fernandez, N. B., Antonides, J. D., Lamb, M. C., Marra, N. J., & DeWoody, J. A. (2015). The reduction of genetic diversity in threatened vertebrates and new recommendations regarding IUCN conservation rankings. *Biological Conservation,***191**, 495-503. <https://doi.org/10.1016/j.biocon.2015.07.025>

Wright, S. (1943). Isolation by distance. *Genetics*, 28(2), 114-138. <https://doi.org/10.1093/genetics/28.2.114>

Wright, S. (1969). *Evolution and the genetics of populations: Vol. 2. The theory of gene frequencies.* University of Chicago Press.

Xu, J. (1995). Analysis of inbreeding depression in *Agaricus bisporus*. *Genetics,***141**, 137-145. <https://doi.org/10.1093/genetics/141.1.137>

Yamanaka, T. (1975). Ecology of *Pseudotsuga japonica* and other coniferous forests in eastern Shikoku. *Memoirs of the National Science Museum,***8**, 119–136 (in Japanese).

Yashima, K. (1994). A geomorphological study of the caldrons in the Seto inland sea. *Report of Hydrographic Researches*, **30**, 237-327 (in Japanese).

Yatoh, K. (1958). Materials for the botanical study on the forest flora of the Kii Peninsula. Analysis and classification of the forest communities. *The bulletin of the Faculty of Agriculture, Mie University*, **18**: 105–167 (in Japanese).

Young, A., Boyle, T., & Brown, T. (1996). The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution,***11**, 413-418. <https://doi.org/10.1016/0169-5347(96)10045-8>

**Data availability statement**

The *Rhizopogon togasawarius* and *Pseudotsuga japonica* SSR genotypes are available at Dryad Data set (Abe et al., 2024).

https://datadryad.org/stash/share/19UnYaq1-H1j16gkDCaEr\_sbBjaVLEo-JLwhP-IJjqA

**Benefit-sharing statement**

Benefits Generated: Our study contributes to the understanding of genetic diversity among crucial forest microorganisms that have been overlooked, thereby offering valuable insights into the conservation of forest ecosystems. While our research focuses solely on samples obtained from Japan, the benefits of this research accrue from sharing our data and results on public databases as described earlier.

**Author contribution**

K.N., M.M., and H.A. conceived and designed the project. K.N., M.M., and H.A. collected samples. H.A. and L.G. developed SSR markers. H.A. genotyped the samples and performed the analyses. H.A. and K.N. wrote the manuscript. All authors read and approved the final version of the manuscript and declare no conflicts of interest.

**Figure legends**

1. **グラフ, ツリーマップ図

   自動的に生成された説明 (b)**

グラフ, 散布図

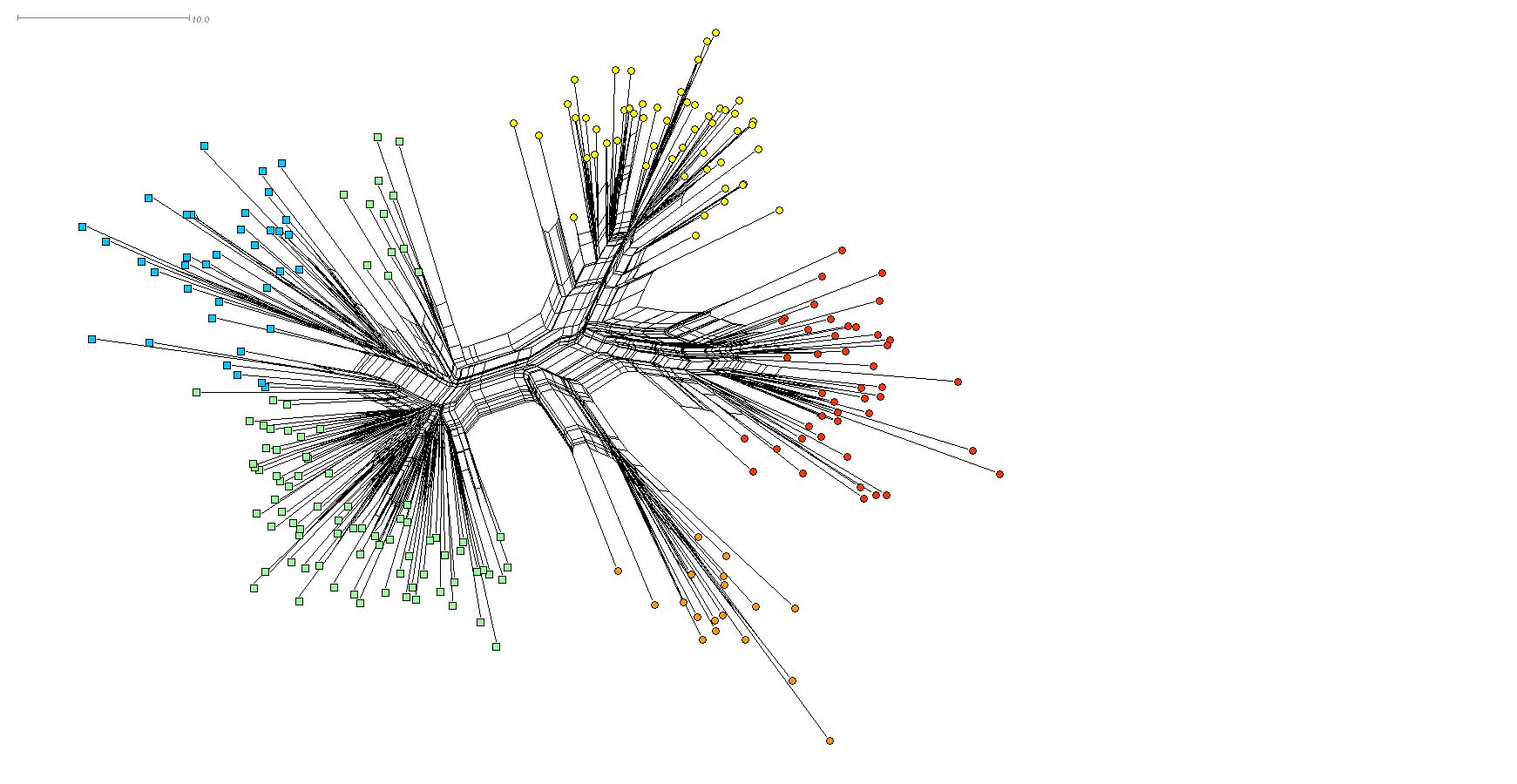
自動的に生成された説明**(c) (d)**

Figure 1. Genetic structure of *Rhizopogon togasawarius.* (a) STRUCTURE bar plot results and (b) geographic distribution; pie chart sizes are proportional to sampling sizes. Different colors represent genetically distinct clusters based on SSR allele compositions. (c) Principal coordinates analysis (PCoA) illustrates genetic distances among individuals; colors correspond to the sampled population and shapes represent different sampling regions. (d) Phylogenetic network tree of *R. togasawarius* individuals; symbols representing individuals are colored and shaped according to Figure 1(c). KW: Kawamatakannon, SN: Sannokogawa, OM: Ohmata, YS: Yasudagoyama, NK: Nishinoko.

1. ダイアグラム

   自動的に生成された説明ダイアグラム

   自動的に生成された説明 (b)

Figure 2: Neighbor-joining trees for population differentiation in *Rhizopogon togasawarius* (a) based on Nei’s *D*A distance with bootstrap support and (b) two scenarios in approximate Bayesian computation and random forest analyses performed using DIYABC-RF version 1.0. KW, SN, OM, YS, and NK represent the origins of the populations; *t*1–*t*4 represent divergence times at individual nodes.

(a)

(b)

Figure 3. (a) Correlations between the geographical distance and fixation index (*F*ST or *G*′ST) of *Rhizopogon togasawarius* populations. Correlations were not statistically significant according to the Mantel test. (b) Correlations of the fixation index (*F*ST or *G*′ST) values between *R. togasawarius* and its host *Pseudotsuga japonica* populations. We detected a relatively strong correlation between the two species, but only the correlation of *F*ST values was statistically significant according to the Mantel test.

Table 1. Sampling sites of *Rhizopogon togasawarius* populations for simple sequence repeat (SSR) analysis

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Population  (Abbrev.) | Latitude  (N°) | Longitude  (E°) | *N*R. | *N*P. | Mean DBH  (cm) |
| KW | 33.91012–15.1 | 135.36737–825 | 53 | 19 | 37.3 |
| SN | 34.26592–41.3 | 136.06755–903 | 44 | 19 | 60.8 |
| OM | 34.01731–691 | 136.08482–412 | 18 | 19 | 77.8 |
| YS | 33.62997–907 | 134.04425–023 | 35 | 20 | 29.2 |
| NK | 33.61039–60912 | 133.96595–294 | 86 | 17 | 61.8 |

*N*R, number of *Rhizopogon togasawarius* individuals; *N*P, number of *Pseudotsuga japonica* individuals. DBH: Diameter at breast height, KW: Kawamatakannon, SN: Sannokogawa, OM: Ohmata, YS: Yasudagoyama, NK: Nishinoko.

Table 2. Characteristics of 20 simple sequence repeat (SSR) markers in *Rhizopogon togasawarius*

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Locus | SSR | Amplification | Primer sequence | | | Size range  (bp) | Total | Heterozygosity | | *F*IS |
| motif | set | (5’–3’) | | | *N*a | *H*o | *H*e |
| *R.ve 1.21* | (ACC)n | set1 | F | HEX | TailB-TGGATGATACTCGGGTACAG | 246–279 | 11 | 0.483 | 0.523 | 0.077 |
| R |  | AGTTCCATCGCCTGAAGTAC |
| *R.ve 1.34* | (CAC)nN | set2 | R | PET | TailD-CTTGATTTACAACATGCAGCG | 208–211 | 2 | 0.147 | 0.165 | 0.111 |
| (CAC)n | R |  | CGAGACCAAGGTGATTTCT |
| *Rtoga* | (GTT)n | set1 | F | FAM | TailA-ACAGACACAGCAGCAGCAAC | 194–200 | 3 | 0.047 | 0.053 | 0.117 |
| R |  | AAACGCATTGGTGGACTGTT |
| *RtogJ* | (CA)n | set2 | F | FAM | TailA-TGGGCTAACTGTGCGTAAGA | 306–326 | 9 | 0.482 | 0.511 | 0.057 |
| R |  | CATGGAAGTGGGTGGTTGAT |
| *RtogQ* | (GTT)nN | set3 | F | HEX | TailB-ATGATGCAGGATCGTCAATG | 306–315 | 4 | 0.225 | 0.307 | 0.267 |
| (GTT)n | R |  | CGCAGGCTTGCTTTATCATC |
| *RtogS* | (CGA)n | set3 | F | FAM | TailA-TTATGTCTCACGGCCCTCTT | 212–218 | 3 | 0.146 | 0.133 | -0.093 |
| (CAA)n | R |  | CGTGATTACCACGGAGATGA |
| *RrosV* | (CAA)n | set4 | F | FAM | TailA-TCGATGGATCATCACCCTCT | 275–320 | 16 | 0.643 | 0.682 | 0.057 |
| R |  | CGGTGCTCTCTCTGATCTCC |
| *RtogX* | (GTT)nN | set4 | F | HEX | TailB-TTGACTCTACCTCCCATGAAAA | 174–180 | 3 | 0.316 | 0.443 | 0.287 |
| (GTT)n | R |  | TCCAAGATGGGATGGAAAAG |
| 3938a | (AAC)n | set5 | F | FAM | TailA-TTGACTTAAGAGCCCGCGAAG | 180–237 | 15 | 0.643 | 0.659 | 0.023 |
| R |  | TGCAGCTTCTTGAAATGCCTG |
| 1037a | (ACG)n | set6 | F | FAM | TailA-TGGCTTCTATGCGCATTGATG | 138–168 | 8 | 0.504 | 0.610 | 0.174 |
| R |  | ATGACGATGACTCAGGATCCG |
| 1651a | (AAC)n | set7 | F | FAM | TailA-GTTCCGACAGCAACAACTCC | 128–164 | 9 | 0.378 | 0.422 | 0.104 |
| R |  | GCCGGAAGCGGTACAAATAC |
| 1693a | (AGG)n | set8 | F | FAM | TailA-CGTTGTTGAGTTCGTCGTTTC | 192–210 | 5 | 0.230 | 0.227 | -0.015 |
| R |  | GCGAGTTTCCCGAATTCTCAG |
| 3267b | (ATC)n | set7 | F | HEX | TailB-TTAAGAGTATGAGCGCGGAGG | 171–219 | 10 | 0.483 | 0.483 | 0.001 |
| R |  | CATCGCTCGTGTCGTTAGAAG |
| 1379b | (AGG)n | set8 | F | HEX | GGTCATTCGGCTCGTACATTC | 144–168 | 7 | 0.377 | 0.417 | 0.096 |
| R |  | CTTCGTTTGGGAGAATGTCGG |
| 1885c | (AGG)n | set6 | F | NED | TailC-AAGGTTAGTACGAGGAGCACG | 202–217 | 6 | 0.404 | 0.480 | 0.159 |
| R |  | GACCTCTTCGTGTATTTGCGG |
| 5992c | (ACG)n | set10 | F | NED | CCGCCGGTATAAATCAAGGAG | 93–126 | 12 | 0.427 | 0.424 | -0.006 |
| R |  | TGACTCTGATGGCTCTGATGG |
| 3629d | (AAG)n | set11 | F | PET | TailD-CATTCTCATAGTGTACGAGGGC | 190–211 | 6 | 0.560 | 0.581 | 0.036 |
| R |  | TTCGTCCATTGTTTCGTCAGG |
| 430d | (ATC)n | set5 | F | PET | TailD-CAGTTAAGCTTCTCCGTTCCG | 202–232 | 10 | 0.629 | 0.698 | 0.098 |
| R |  | ACACGACTTGCAGACACTTAC |
| 1332d | (ATC)n | set6 | F | PET | TailD-TGTCCAGCATCCATACAACG | 180–246 | 18 | 0.553 | 0.569 | 0.028 |
| R |  | TCATCCCAGCAGTCTTCCTTC |
| 2370d | (AGG)n | set7 | F | PET | TailD-GATGGCTCAGGTGACAATGAG | 161–197 | 10 | 0.588 | 0.603 | 0.024 |
| R |  | TGTTTCTGGTCCGCTTGTTTC |

*H*e, expected heterozygosity; *H*o, observed heterozygosity; *N*a, number of alleles scored.

We multiplexed 2–3 SSR markers in PCR followed by fragment analysis, as shown in the amplification set column.

Table 3. Population genetic indices of *Rhizopogon togasawarius*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Population | *A*e | *H*o | *H*e | *A*r | *F*IS |
| KW | 1.74 (±0.13) | 0.324 (±0.043) | 0.364 (±0.046) | 2.94 (±0.29) | 0.120 (±0.045)\* |
| SN | 2.31 (±0.27) | 0.395 (±0.062) | 0.437 (±0.063) | 3.99 (±0.49) | 0.106 (±0.041)\* |
| OM | 2.21 (±0.19) | 0.428 (±0.057) | 0.460 (±0.056) | 3.10 (±0.26) | 0.098 (±0.057) |
| YS | 2.37 (±0.19) | 0.476 (±0.041) | 0.524 (±0.041) | 3.76 (±0.28) | 0.106 (±0.034)\* |
| NK | 2.52 (±0.31) | 0.444 (±0.061) | 0.464 (±0.065) | 4.00 (±0.50) | 0.049 (±0.018)\* |
| Total | 2.17 (±0.10) | 0.400 (±0.024) | 0.438 (±0.024) | 5.27 (±0.55) | - |

Values represent the means across 20 SSR loci (±standard error).

*A*e, effective number of alleles of each population; *A*r, allelic richness; *F*IS, inbreeding coefficient; *H*e, expected heterozygosity; *H*o: observed heterozygosity. Asterisks indicate significant deviation from zero (*P* < 0.05). KW: Kawamatakannon, SN: Sannokogawa, OM: Ohmata, YS: Yasudagoyama, NK: Nishinoko.

Table 4. Effective population size (*N*e) and bottleneck estimation in five *Rhizopogon togasawarius* populations

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Effective population size | | Bottleneck (T.P.M) | |  |
| Population | *N*e (95% CI) | Expected loci with excess | Observed loci with excess | Wilcoxon (*p*–Value) |
| KW | 9.0 (5.4–15.2) | 9.80 | 6 | 0.951 |
| SN | 15.5 (10.1–26.0) | 9.74 | 5 | 0.998 |
| OM | 23.5 (13.1–60.4) | 10.40 | 11 | 0.114 |
| YS | 30.3 (17.9–71.0) | 11.25 | 8 | 0.813 |
| NK | 58.0 (26.9–1682.7) | 10.40 | 4 | 0.983 |

No significant bottleneck was detected in *R. togasawarius* populations. KW: Kawamatakannon, SN: Sannokogawa, OM: Ohmata, YS: Yasudagoyama, NK: Nishinoko.

Table 5. Pairwise *F*ST and *G*′ST values between populations of *Rhizopogon togasawarius*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Population | KW | SN | OM | YS | NK |
| KW | – | 0.388\* | 0.482\* | 0.515\* | 0.445\* |
| SN | 0.152\* | – | 0.445\* | 0.540\* | 0.499\* |
| OM | 0.191\* | 0.163\* | – | 0.508\* | 0.460\* |
| YS | 0.188\* | 0.200\* | 0.176\* | – | 0.208\* |
| NK | 0.177\* | 0.198\* | 0.171\* | 0.075\* | – |

Lower and upper diagonal values indicate pairwise *F*ST and *G*′ST, respectively. Asterisks indicate significant *F*ST and *G*′ST values (*P* < 0.05). KW: Kawamatakannon, SN: Sannokogawa, OM: Ohmata, YS: Yasudagoyama, NK: Nishinoko.

Table 6. Results of DIYABC-RF for *Rhizopogon togasawarius* populations, including (a) scenario choice and (b) divergence time estimation under scenarios 1 (±standard deviation)

(a)

|  |  |  |  |
| --- | --- | --- | --- |
| Prior error rate | Posterior probability | **Scenario 1** | Scenario 2 |
| 0.185 (±0.002) | 0.722 (±0.024) | **55.2% (±2.1)** | 44.8% (±2.1) |

(b)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Parameter | Posterior point estimates of | | Global NMAE | Local NMAE |
|  | Median | 90% CI | Median | Median |
| *t*1 | 1512.2 (±35.8) | 648.6–3438.4 (±27.0–177.9) | 0.321 (±0.021) | 0.292 (±0.084) |
| *t*2 | 3248.1 (±69.7) | 1443.6–6303.9 (±65.7–274.1) | 0.273 (±0.009) | 0.355 (±0.116) |
| *t*3 | 4969.0 (±82.1) | 2209–8187.2 (±102.4–87.1) | 0.228 (±0.008) | 0.334 (±0.065) |
| *t*4 | 6038.3 (±67.6) | 2886.7–9550.6 (±86.0–32.6) | 0.154 (±0.004) | 0.210 (±0.053) |

NMAE: Normalized mean absolute error, CI: confidence interval